CHAPTER 1

Biosensors and Bio-Based Methods for the Separation and Detection of Foodborne Pathogens

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Contents

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Abstract

The safety of our food supply is always a major concern to consumers, food producers, and regulatory agencies. A safer food supply improves consumer confidence and brings economic stability. The safety of foods from farm-to-fork through the supply chain continuum must be established to protect consumers from debilitating, sometimes fatal episodes of pathogen outbreaks. The implementation of preventive strategies like hazard analysis critical control points (HACCP) assures safety but its full utility will not be realized unless supportive tools are fully developed. Rapid, sensitive, and accurate detection methods are such essential tools that, when integrated with HACCP, will improve safety of products. Traditional microbiological methods are powerful, error-proof, and dependable but these lengthy, cumbersome methods are often ineffective because they are not compatible with the speed at which the products are manufactured and the short shelf life of products. Automation in detection methods is highly desirable, but is not achievable with traditional methods. Therefore, biosensor-based tools offer the most promising solutions and address some of the modern-day needs for fast and sensitive detection of pathogens in real time or near real time. The application of several biosensor tools belonging to the categories of optical, electrochemical, and mass-based tools for detection of foodborne pathogens is reviewed in this chapter. Ironically, geometric growth in biosensor technology is fueled by the imminent threat of bioterrorism through food, water, and air and by the funding through various governmental agencies.

I. INTRODUCTION

Food safety and food biosecurity continued to draw the attention of consumers, food manufacturers, and producers. Foodborne pathogen statistics show slight declines in the number of cases but increased number of outbreaks (Lynch *et al*., 2006), and product recalls continue to place a huge economic burden on producers and processors. The elimination of pathogens from raw unprocessed products had been the focus to reduce burdens before the products are transported to the processing plant. On-farm, pathogen-controlling strategies will help achieve that goal. However, the presence of pathogens in ready-to-eat products is a serious concern since these products generally do not receive any further treatment before consumption. In fact many recent foodborne outbreaks resulted from consumption of undercooked or processed ready-to-eat meats (hotdogs, sliced luncheon meats, and salami), dairy products (soft cheeses made with unpasteurized milk, ice cream, butter, etc.), or

minimally processed fruits (apple cider, strawberries, cantaloupe, etc.) and vegetables (sprouts, lettuce, etc.) (Altekruse *et al*., 2006; CDC, 2006; Doyle and Erickson, 2006; Lynch *et al*., 2006; Sivapalasingam *et al*., 2004). Food animals and poultry are the most important reservoirs for many of the foodborne pathogens. Therefore, meat, milk, or egg products may carry *Salmonella*, *Campylobacter*, *Listeria*, or *Escherichia coli* O157:H7 organisms. These products should be tested before retail distribution. Animal by-products, such as feed supplements, may also transmit pathogens to other animals [for example, *Salmonella*, bovine spongiform encephalopathy (BSE)] (Dormont, 2002). The application of untreated manure onto farmland may contaminate soil or water and eventually transmits microbes to fruits or vegetables (Brandl, 2006; Solomon *et al*., 2002). Seafoods are another potential source of pathogens, such as *Vibrio*, *Listeria*, *Yersinia*, *Salmonella*, *Shigella*, *Clostridium*, *Campylobacter*, and Hepatitis A (Carter, 2005; Feldhusen, 2000).

Immunologically challenged populations such as the elderly, infants, person with malignant cancer, AIDS, or organ transplants are at higher risk than healthy ones for some of the foodborne pathogens (Trevejo *et al*., 2005). The infectious doses of many of these pathogens are very low (~10–1000 bacterial cells) (Balbus and Embrey, 2002). Detection technologies, both traditional and rapid, have helped ensure food safety, but ongoing concern with intentional administration of harmful microorganisms or toxins to food or water demands further improvement in detection technologies for fast time-to-result with a high degree of accuracy. In recent years, there has been an explosion of research activities in the area of sensor development with a primary focus on the biologically significant molecules including pathogenic microorganisms.

Culture-based methods are considered a gold-standard for foodborne pathogen detection (Gracias and McKillip, 2004; Swaminathan and Feng, 1994), and this serves as a foundation for some of the modern-day rapid methods. The analysis of foods for the presence of both pathogenic and spoilage bacteria is a standard practice for ensuring food safety and quality. Traditional culture methods rely on specific microbiological media to isolate and enumerate viable bacterial cells in foods. These methods usually consist of five steps involving pre-enrichment, selective enrichment, selective plating, biochemical tests, and serological tests. The pre-enrichment step is beneficial since this step not only increases the populations of the target organism but also allows recovery of sublethally injured or stressed bacteria resulting from exposure to processing and storage conditions of heating, drying, freezing, cooling, preservatives, etc. Injured microbes are capable of recovering in food and causing disease in humans. Selective antimicrobial compounds are then added to the medium in the beginning or within 2–4 h of culturing to suppress the growth of competitive microorganisms. An aliquot of culture from

the enrichment liquid is then plated onto selective or differential agar plates for the isolation of pure cultures. The identity of the isolated cultures is determined by phenotypic analysis of virulence traits or by biochemical characterization (metabolic fingerprinting). Culture methods are usually very sensitive, relatively inexpensive, and can give both qualitative and quantitative information on the number and the nature of the microorganisms. However, these methods are labor intensive and lengthy, requiring 5–7 days. Rapid and sensitive methods are highly desirable during implementation of hazard analysis critical control points (HACCP), especially in the food-processing plant for monitoring pathogens in raw materials, ready-to-eat food products, and to verify manufacturing process control. Bacteria-specific generic rapid methods such ATP-based luminescence and total plate count (APC) are also needed for monitoring cleaning and hygienic practices employed in a processing plant.

Biosensor-based tools continue to capture the imaginations of researchers and users for their potential for the sensitive detection of pathogens in automated or semiautomated instruments in near real time. Broadly, pathogen detection is centered on four basic physiological or genetic properties of microorganisms: metabolic patterns of substrate utilization, phenotypic expression analysis of signature molecules by antibodies, nucleic acid analysis, and the analysis of the interaction of pathogens with eukaryotic cells (cytopathogenic effects). Many of today's popular commercially available rapid methods use culture-based methods coupled with automated or semiautomated nucleic acid-, antibody-, or substrate utilization-based methods to obtain results in 24–72 h. Interestingly, many of the modern-day biosensor-based methods are developed utilizing one of the above four principles or combinations of some sort. However, antibody-based methods are the most popular because of their versatility, convenience, and relative ease in interpretation of the data. It is interesting to note that a majority of biosensors use antibody for capture and detection of the target analyte.

II. SEPARATION AND CONCENTRATION OF PATHOGENS FROM SAMPLES

For pathogen detection, the sample preparation step is crucial. It is even more important when biosensor tools are employed for the detection. Food or environmental samples are highly complex consisting of fats, carbohydrates, proteins, salts, antimicrobial preservatives, etc., and moreover, the target pathogen numbers are generally very low. Thus, highly efficient pathogen separation and concentration strategies are needed to

achieve successful detection of pathogens and to avoid false-negative results. Several strategies including antibody-based and physical- and chemical-based separation and concentration methods have been developed for separation and concentration of pathogens from various sample matrices (Stevens and Jaykus, 2004). Antibody-based methods include immunomagnetic separation (IMS) (discussed in Section II.A.1), while the physical and chemical methods include centrifugation, filtration, chromatographic separation, and dielectrophoresis (DEP) (Chen *et al.*, 2005a,b; Li and Bashir, 2002; Stevens and Jaykus, 2004).

A. Antibody, a key molecule in bioseparation and detection

Antibodies are widely used for pathogen capture, concentration, and detection purposes (Liddell, 2005). The antibody-based assay methods are simple, less cumbersome, and easy to interpret and these methods allow detection of not only intact microbial cells but also their secreted toxins or by-products (Bhunia, 1997; Macario and De Macario, 1988). However, in some cases, antibody-based methods may not be able to differentiate live from dead cells. Immunoassays coupled with an active culturing method can overcome this problem. Additionally, microbes are routinely exposed to stress conditions such as acidic or alkaline pH, osmotic stress (salt), antimicrobial preservatives, storage temperatures, and heat shock conditions in food, which may alter their morphology and affect physiology resulting in aberrant antigen expression, which could weaken signals during antibody-based detection (Geng *et al.*, 2003, 2006a, Hahm and Bhunia, 2006).

The availability of an antigen-specific antibody is the key to the success of immunoassays. Furthermore, the binding affinity and avidity of antibodies are important properties, which should be thoroughly characterized before employing antibodies for specific applications. Polyclonal antibodies (PAbs) contain an assortment of antibody molecules recognizing different antigens and epitopes, and therefore may show some cross-reactions with antigens from different microbes. PAbs can be made epitope-specific for improved detection. The quality of PAbs may vary from batch to batch, which could affect the end-result. On the other hand, monoclonal antibody (MAb) is homogeneous and highly specific. Since MAb is produced by a single preselected clone (hybridoma line), it is always highly specific toward an antigen. Carefully designed experiments employing both PAbs and MAbs can provide the highest specificity in an immunoassay or immunosensor applications with reproducible and desirable results.

1. Immunomagnetic separation

Antibodies are also an integral part of sample preparation allowing the specific capture and concentration of bacteria from complex food matrices for detection by various methods. IMS methods use magnetic particles coated with ligands, including antibodies to purify target molecules from a mixture. Most of the particles used for these separations are superparamagnetic, that is, they only exhibit magnetic properties in the presence of an external magnetic field. They can be easily removed from a suspension by a magnetic separator (Safarik *et al.*, 1995). Since there is usually no magnetic remmnance, the particles are not attracted to each other and therefore they can be easily suspended into a homogeneous mixture in the absence of any external magnetic field.

The separation process for the purification of target cells using magnetic particles and magnetic separators usually consists of two fundamental steps. First, the suspension containing the cells of interest is mixed with immunomagnetic particles. Interaction of the target cells and the beads occurs during the incubation step (usually no longer than 30–60 min). Then the magnetic complex is separated using an appropriate magnetic separator, and the supernatant is discarded. Second, the magnetic complex is washed several times to remove unwanted contaminants. In this form, the selected cells with attached magnetic particles can be used for the further experiments.

The most common magnetic carriers are the Dynabeads® (Dynal, Inc., Oslo, Norway) with diameter ranging from 2.8 to 4.5 μm. These are polystyrene beads coated with iron oxide and antibodies are generally immobilized using streptavidin and biotin chemistry. Immunomagnetic beads have been used for concentration and separation of selected microorganism from environmental samples (Mitchell *et al.*, 1994) or foods (Skjerve *et al.*, 1990). Either direct- or indirect-IMS can be used for recovery of the target organism. In the direct approach, the target organism is mixed with the magnetic particles that are coated with antibody specific for the organism. When the particles come in contact with the bacterial cells, they attach via the primary antibody. Once the particles are concentrated, the remaining solution is discarded and only the bound bacteria are recovered.

In the indirect approach, the primary antibody is added to the suspension and allowed to attach to the target organism. Then the magnetic particles, coated with a secondary antibody specific for the primary antibody, are added and allowed to attach to the primary antibody. The magnetic particle complexes are then separated using the magnetic concentrator and the solution is removed, with only the bound bacteria remaining. The bacteria collected do not need to be detached since they are viable and can multiply as long as a sufficient amount of media is provided (Torensma *et al.*, 1993).

Although experimentation for each specific application must be done to determine the number of immunomagnetic particles needed for capture, the general guidelines for the use of small particles (<5 μm sized) are in the ratio of 3:1–20:1 (beads: target cells) (Safarik *et al.*, 1995). The incubation time could vary from 10 to 60 min. Longer incubations tend to improve capture efficiency; however, they may result in considerable nonspecific binding. Washing steps generally reduce nonspecific binding and the use of mild detergents, such as Tween-20, during initial incubation and subsequent washing steps further decreases nonspecific binding (Okrend *et al.*, 1992; Safarik *et al.*, 1995).

One of the major drawbacks of using IMS is that there is a high likelihood that the magnetic particles will capture more than one of the target cells or that the particles or cells form aggregates, thus resulting in formation of only one colony when plated on a solid agar surface (Skjerve *et al.*, 1990). Using fluorescence and scanning electron microscopy, it has been estimated that, depending on the bead size, each colony formed from an aggregate may contain up to six bacteria (Safarik *et al.*, 1995).

Immunomagnetic beads have been used for capture of *E. coli* O157:H7 (Chapman and Ashton, 2003; Evrendilek *et al.*, 2001; Yu and Bruno, 1996), *Salmonella* (Favrin *et al.*, 2001; Jordan *et al.*, 2004; Rijpens *et al.*, 1999; Trkov *et al.*, 1999), and *Listeria* (Fluit *et al.*, 1993; Kaclikova *et al.*, 2001; Uyttendaele *et al.*, 2000). Sometimes, the capture efficiency is highly variable, which depends on the pathogen and the quality of the antibodies used. In recent years, application of IMS coupled with PCR assays are showing very promising results for the detection of *E. coli* O157:H7 (Fu *et al.*, 2005), *Salmonella enterica* (Jenikova *et al.*, 2000; Mercanoglu and Aytac, 2002; Mercanoglu and Griffiths, 2005), and *Listeria monocytogenes* (Amagliani *et al.*, 2006; Ueda *et al.*, 2006). The detection limit for IMS with PCR was 1 cfu/1–25 g of sample following enrichment for *L. monocytogenes* (Hudson *et al.*, 2001). IMS with flow cytometry had a detection limit of 3×10^4 (Jacobsen *et al.*, 1997; Jung *et al.*, 2003a) and with colony-blot it was 1–10 cfu/25 g (Wieckowska-Szakiel *et al.*, 2002). IMS has been used in conjunction with an immunofluorescence assay for successful detection of *Listeria* cells as low as 10^3 cfu/ml (Duffy *et al.*, 1997). The major drawbacks of the IMS-based assays are the requirement of an enrichment and a sample clean up step. Microbeads (typical size is ca. 50–200 nm) coated with a polyclonal anti-*Listeria* antibody to separate *L. monocytogenes* for flow cytometry has been used (Jacobsen *et al.*, 1997). In that experiment, the recovery rate of pure culture of *L. monocytogenes* was about 91% when cell concentration was 10^9 cells/ml. When the cell concentration was lowered to between 10^4 and 10^8 cells/ml, the recovery rate was between 40% and 70%.

A modified immunoseparation method employing agarose beads called Immunobeads was also used for *Listeria* capture. Immunobeads are Protein A conjugated agarose beads. In this experiment, Immunobeads were covalently linked with a MAb showing a high degree of specificity for *L. monocytogenes* (Gray and Bhunia, 2005). Immunobeads were able to capture ~10% of *L. monocytogenes* cells at initial cell concentration levels of 1–100 cfu/100 ml from hotdog extracts in 12–18 h. The captured *Listeria* cells were then tested by a cytotoxicity assay for specific detection of *L. monocytogenes* (Gray and Bhunia, 2005).

A flow-through immunocapture system called Pathatrix® (Matrix MicroScience Ltd., Cambridgeshire, UK) has been developed that can process up to 250 ml of sample homogenates. The liquid is pumped through a tubing system in which the sample passes over an area where IMS beads are trapped using a magnet and the captured cells are collected in a small sample volume. This system has been validated for capture and detection of *Salmonella*, *E. coli* O157:H7, and *Listeria* species (Prentice *et al.*, 2006).

An automated immunomagnetic capture system called BeadRetriever (Dynal Biotech Ltd., Wirral, UK) has been used for pathogen capture and concentration. This system works on the inverse magnetic particle processing principle, where the paramagnetic beads bound to a magnetic rod are moved from tube to tube containing specific reagents, such as test samples containing target pathogen, washing solutions, enzyme conjugated pathogen specific antibody, and substrates. This system has been used for the detection of *E. coli* (Chapman and Cudjoe, 2001; Fegan *et al.*, 2004; Reinders *et al.*, 2002), *Salmonella* (Duncanson *et al.*, 2003), and *L. monocytogenes* (Amagliani *et al.*, 2006).

III. BIOSENSOR-BASED DETECTION METHODS

Biosensors are devices that detect biological or chemical recognition complexes in the form of either antigen–antibody, enzyme–substrate, or receptor–ligand, placed in proximity to a transducer that generates a signal. Biosensor-based technologies have been increasingly used in the development of methods to sensitively detect foodborne pathogens (Anderson and Taitt, 2005; Baeumner, 2004; Bhunia and Lathrop, 2003; Deisingh and Thompson, 2004; Geng and Bhunia, 2007; Iqbal *et al.*, 2000; Ivnitski *et al.*, 1999; Leonard *et al.*, 2003; Rasooly and Herold, 2006). The pathogen-detecting biosensor market is estimated to be $563 million and grows at a compounded annual rate of 4.5%, with the food processing making up $192 million of that amount (Alocilja and Radke, 2003).

The application of biosensors for pathogen detection from food samples is challenging because of the complex nature of food matrices, which

consist of fats, proteins, carbohydrates, and additives with different acidities, salt concentrations, and colorings. Application of nano/micro technology to detect pathogens from such complex systems presents numerous challenges. This is further complicated by the fact that the populations of target microorganisms are often extremely small compared to the indigenous ones. Therefore, exceptionally intelligent strategies should be employed for detection of such low numbers of pathogens directly from food. Varieties of biosensor-based methods have been and continued to be developed and those are grouped as electrochemical (impedance-based, amperometric), optical (fiber optic, surface plasmon resonance), thermometric (thermister, pyroelectric), and mass based (piezoelectric, surface acoustic). Among these, optical biosensors appear to be the most widely used sensors for foodborne pathogens because of their sensitivity, available instrumentation, and relative ease of data interpretation. Besides optical sensors, electrochemical and mass-based sensors are also used for foodborne pathogens as discussed in this chapter.

A. Fiber optic biosensor

Fiber optic biosensor is one of the first commercially available optical biosensors, marketed by Research International (Monroe, WA) for the detection of foodborne as well as pathogens of biosecurity importance. The manual version of the instrument is called Analyte 2000 and the portable semiautomated version is called RAPTOR^{TM}.

The basic principle of the fiber optic sensor is that when light propagates through the core of the optical fiber (waveguide), it generates an evanescent field outside the surface of the waveguide. When fluorescent-labeled analytes such as pathogens or toxins bound to the surface of the waveguide, are excited by the evanescent wave generated by a laser (635 nm), and emit fluorescent signal (Bhunia *et al.*, 2007; Taitt *et al.*, 2005), the signal travels back through the waveguide in high order mode to be detected by a fluorescence detector in real time (Fig. 1.1) (Anderson *et al.*, 1992, 1996; Hirschfeld, 1985; Hirschfeld *et al.*, 1984; Marazuela and Moreno-Bondi, 2002; Mehrvar *et al.*, 2000).

The waveguides are generally made up of polystyrene fibers or glass slides, and the latter one is also called planar waveguide. On the planar waveguides, multiple analytes could be detected in a patterned microarray format (Ligler *et al.*, 2003). To detect the analytes, a laser of 635 nm is used and two-dimensional imaging systems are employed where charge-coupled device (CCD) (Golden *et al.*, 2005; Wadkins *et al.*, 1997) or complimentary metal oxide silicon (CMOS) chips (Moreno-Bondi *et al.*, 2003; Vo-Dinh *et al.*, 1999) were used for acquiring images. Recently, a portable automated device called Naval Research Laboratory (NRL) array

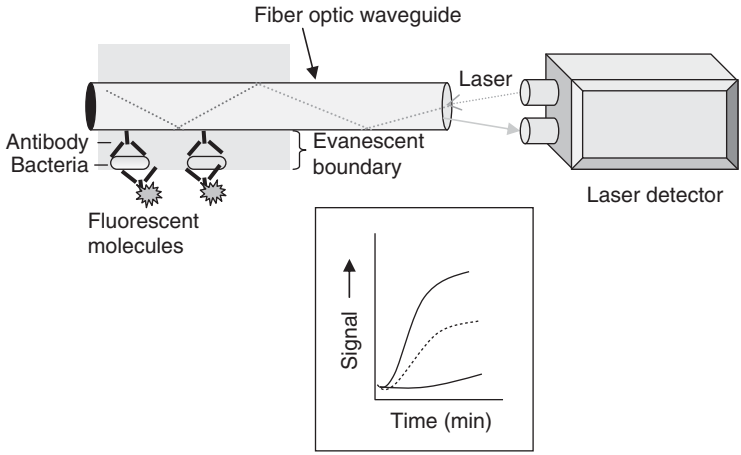

FIGURE 1.1 Schematic drawing showing fiber optic waveguide-based detection of pathogens. Graph showing the real-time rise in signal as the fluorescent-labeled antibody binds to the antigen forming a sandwich.

biosensor was built that has supporting microfluidic system and data acquisition system for onsite use (Golden *et al.*, 2005).

Analyte 2000 (Research International, Monroe, WA) and the semiautomated RAPTOR™ (Research International) use four polystyrene optical fibers (4-cm long) and a laser source of 635 nm. Analyte 2000 can be used with 1–4 separate fibers while the RAPTOR™ holds a coupon that holds four fibers together so that a maximum of four analytes can be detected at one time from a sample. Moreover, RAPTOR™ is built with a sample injection port, microfluidic channels, laser diode, and computer interface for data analysis. The fibers can be reused until a positive response is recorded. The assay principle for evanescent wave biosensors is based on a sandwich immunoassay. First, capture antibodies or receptor molecules are immobilized onto the optical waveguides. Upon binding of an analyte, a Cyanine 5 (Cy5)-labeled or Alexa-Fluor 647-labeled antibody is then added (Anderson and Nerurkar, 2002; Marazuela and Moreno-Bondi, 2002). The laser is launched into the proximal end of the optical waveguides and the analyte bound fluorescent molecules within several hundred nanometers of the waveguides are excited by an evanescent field. Then a portion of their emission energy reemits into the waveguides. A photodiode allows for quantitation of the collected emission light at wavelengths of 670 to 710 nm (Anderson *et al.*, 1996).

Antibodies are commonly used for the capture of target molecules on the waveguides and they are immobilized by either physical adsorption or through a self-assembly monolayer (SAM). In the former situation,

antibodies are nonspecifically bound to a polystyrene surface through ionic interactions and aid in capture. Poly-L-lysine or gluteraldehyde are also used to facilitate immobilization. The physical adsorption method, though simple, often results in a greater variation in signal output. Solvents, pH changes, or salts may affect the antibody cross-linking and thus may affect reproducibility. Antibodies are also immobilized on the waveguides through the formation of a SAM in which avidin/biotin chemistry, Protein A, and Protein G are used. Avidin derivatives (streptavidin, neutravidin) can be covalently linked onto the surface or can be bound through a biotinylated protein layer such as bovine serum albumen. Biotinylated antibody is then allowed to bind to the avidin layer. Layers of Protein A or Protein G also could be covalently linked onto the waveguides for immobilization of the IgG subclass of antibodies (Anderson *et al.*, 1997). Ligand-specific receptor molecules have also been used on sensor surfaces for detection of specific toxins. For example, an acetylcholine receptor has been used to detect α-bungarotoxin and α-cobratoxin (Rogers *et al.*, 1989) and gangliosides GM1 and GT1b were used in microarray format for capture of cholera and tetanus toxin (Fang *et al.*, 2003), respectively.

Use of optical fiber biosensors for real-time detection of biowarfare agents (BWA) especially those of bacterial cells, toxins, or spores in the air, soil, or environment has been investigated by the Naval Research Laboratory (Taitt *et al.*, 2005). In addition, many laboratories are also employing fiber optic biosensors for detection of wide varieties of foodborne pathogens, which are discussed below.

Staphylococcus aureus produces 17 types of enterotoxins, which are responsible for food poisoning and fatal toxic shock syndrome (Stewart, 2005). Among these, staphylococcal enterotoxin (SE), type B (SEB) is considered as one of the most potent biothreat agents. Fiber optic biosensors have been developed for the detection of SEB (King *et al.*, 1999; Tempelman *et al.*, 1996). Later, fiber optic-based RAPTOR^{TM} using Alexa Fluor-labeled detection antibody was able to sensitively detect SEB alone (Anderson and Nerurkar, 2002) or SEB in combination with other biothreat agents: *Bacillus anthracis*, *Francisella tularensis*, and *Bacillus globigii* spores (Jung *et al.*, 2003b). A combination of surface plasmon resonance (SPR) and a side-polished single-mode optical fiber with a thin metal layer was developed for the detection of SEB at nanogram quantities (Slavik *et al.*, 2002). An array biosensor patterned on glass slides was also able to detect 12 different analytes including several bacterial pathogens and some toxins: SEB, ricin, cholera toxin, botulinum toxin, and fumonosin each at 0.5 ng/ml (Ligler *et al.*, 2003). Using the NRL array biosensor, SEB was also detected at 0.1 ng/ml when spiked into various food products: tomatoes, sweet corn, green beans, mushrooms, and tuna (Sapsford *et al.*, 2005). *Clostridium botulinum* neurotoxin was also

detected using a fiber optic sensor or NRL array biosensor (Ogert *et al.*, 1992; Sapsford *et al.*, 2005) from various spiked foods at 20 ng/ml concentrations.

Seo *et al.* (1999) used a planar optic biosensor that measures the phase shift variation in refractive index due to antigen binding to antibody. In this method, they were able to detect *S. enterica* serovar Typhimurium with a detection limit of 1×10^5 cfu/ml. When chicken carcass fluid was inoculated with 20 cfu/ml, the sensor was able to detect this pathogen after 12 h of nonselective enrichment. A compact fiber optic sensor was also used for detection of *S.* Typhimurium at a detection limit of 1×10^4 cfu/ml (Zhou *et al.*, 1997, 1998); however, its efficacy with food samples is unproven. Later, Kramer and Lim (2004) used the fiber optic sensor, RAPTOR^{TM}, to detect this pathogen from spent irrigation water for alfalfa sprouts. They showed that the system can be used to detect *Salmonella* spiked at 50 cfu/g seeds. An evanescent wave-based multi-analyte array biosensor (MAAB) was also employed for successful testing of chicken excreta and various food samples (sausage, cantaloupe, egg, sprout, and chicken carcass) for *S.* Typhimurium (Taitt *et al.*, 2004). While some samples exhibited interference with the assay, overall, the detection limit for this system was reported to be 8×10^3 cfu/g.

An evanescent fiber optic biosensor was also developed and used successfully for *E. coli* O157:H7. DeMarco and his colleagues (DeMarco and Lim, 2002; DeMarco *et al.*, 1999) were able to detect 3–30 cfu/ml in spiked ground beef samples. They also compared the detection limit for two types of fiber optic waveguides. For a silica-based waveguide, they were able to detect 9×10^3 cfu/g, while for a polystyrene waveguide, the limit was 5.2×10^2 cfu after overnight enrichment of samples (DeMarco and Lim, 2002; DeMarco *et al.*, 1999). More recently, Geng *et al.* (2006b) used Alexa Fluor-647-labeled antibody for the sensitive detection of *E. coli* O157:H7 from ground beef after only 4 h of enrichment with an initial inoculation level of 1 cfu/g. In a report, Maraldo *et al.* (2006) used a fiber optic sensor to directly monitor bacterial growth on the tapered fiber. They immobilized GFP-expressing *E. coli* on the fiber using poly-L-lysine. Growth rapidly decreased the transmission of the evanescent signal.

A fiber optic sensor was also developed for successful detection of *Listeria* species. Strachan and Gray (1995) were able to detect PCR-amplified products of *Listeria* spp. on a fiber optic sensor. In this method, DNA oligomers (40 bp) derived from the flagellin gene were immobilized onto the glass fibers using gluteraldehyde. PCR-amplified products internally labeled with FAM (Fluorescence amadite) were allowed to hybridize for detection by a solid-state sensor. A fiber optic immunosensor was designed using a MAb for both capture and detection in a sandwich format, which produced a positive signal from *L. monocytogenes* cells ($\sim 10^8$ cfu/ml) with a significantly lower signal from *Listeria innocua*

(Bhunia *et al.*, 2001). Later, the sensitivity of the assay was greatly improved by using a *Listeria* genus-specific PAb as the capture antibody and a MAb as the detection antibody (Geng *et al.*, 2004). The detection limit for *L. monocytogenes* was established to be 1×10^3 cfu/ml. The sensor was highly specific and was able to detect the target pathogen at the 1×10^4 cfu/ml level from artificially inoculated meat samples in the presence of natural background microflora. Recently, the RAPTOR^{TM} was used in an assay to detect *L. monocytogenes* employing a flow-through antibody immobilization method on waveguides and the detection limit was determined to be 1×10^3 and 5×10^5 cfu/ml in frankfurter samples (Nanduri *et al.*, 2006). Tims *et al.* (2001) were able to detect pure cultures of *L. monocytogenes* with the detection limit of 4.1×10^8 cfu/ml. They concluded that the quality of antibodies is the key in improving sensitivity.

This NRL sensor was used for the rapid detection of *Campylobacter jejuni* and small toxins, including several mycotoxins [ochratoxin A, fumonisin B, aflatoxin B_1, and deoxynivalenol (DON)] from food products (Ngundi *et al.*, 2005, 2006; Sapsford *et al.*, 2006). They used a sandwich immunoassay format to detect *C. jejuni* in milk and yogurt and a competitive immunoassay format to detect the mycotoxins.

Recently, a fiber optic microsphere-based DNA array was also reported for the detection of multiple BWA including *B. anthracis*, *Yersinia pestis*, *F. tularensis*, *Brucella melitensis*, *C. botulinum*, *Vaccinia virus*, and one biological warfare agent simulant, *Bacillus thuringiensis* Kurstaki in a multiplexed format (Song *et al.*, 2006). This system was validated with contaminated sewage samples and they suggested that the system could be integrated into a portable instrument for BWA detection.

B. SPR sensor

SPR is a phenomenon that occurs during optical illumination of a metal surface, and it can be used for biomolecular interaction analysis. Receptors or antibodies immobilized on the surface of a thin film of a precious metal (gold) deposited on the reflecting surface of an optically transparent waveguide are used to capture the target analyte. The sensing surface is located above or below a high index-resonant layer and a low index-coupling layer (Fig. 1.2). When a visible or near-infrared radiation (IR) is passed through the waveguide in such a way, it causes an internal total reflection on the surface of the waveguide. At a certain wavelength in the red or near-IR region, the light interacts with a plasma or cloud of electrons on the high-index metal surface, and the resonance effect causes a strong absorbance. The exact wavelength of this absorption depends on the angle of incidence, the metal, the amount of capture molecules immobilized on the surface, and the surrounding material. The presence of

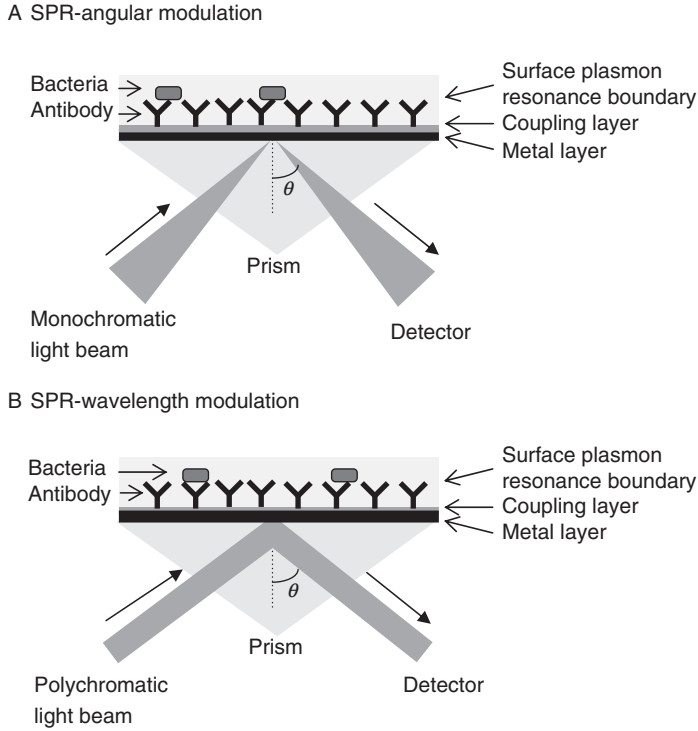

FIGURE 1.2 Schematic drawing showing SPR sensor with different configurations: (A) angular modulation and (B) wavelength modulation as described by Homola *et al.* (2002).

ligands or antigens interacting with the receptor or antibody causes a shift in the resonance to longer wavelengths, and the amount of shift can be related to the concentration of the bound molecules.

Surface immobilization of the capture molecules follows standard procedures that are commonly practiced in many biosensor applications and some are discussed in the previous section. Layers of carboxymethyl dextran, Protein A or Protein G, streptavidin-coated surface, or EDC [*N*-ethyl-*N*-(diethylaminopropyl) carbidimide]/NHS (*N*-hydroxysuccinimide)-based amine coupling through amide bond are used for protein (antibody, receptor, etc.) cross-linking.

SPR-based sensors are governed by two basic principles: wavelength interrogation and angle interrogation (Fig. 1.2, Homola *et al.*, 2002). Wavelength interrogation uses a fixed angle of incidence but measures spectral changes, while in angle interrogation, a fixed wavelength is used but the angle of reflectance is monitored. Most of the commercial SPR systems are operated based on the angle interrogation mode such as the prism-based BIACORE (Biacore AB, Uppsala, Sweden), Spreeta™ (Texas

Instrument, Dallas, TX), SPR spectroscope (Multiscop^{TM}, Optrel GbR, Germany), Reichert SR7000 (Reichert Analytical Instruments, Depew, NY), and resonant mirror based IAsys (Thermo Labsystems, Cambridge, UK).

There are several advantages of SPR-based sensors. They allow real-time or near real-time (under 30 min) detection of binding events between two molecules. The detection system is label free, thus eliminating the need for additional reagents, assay steps, and time. The sensor can be reused for the same analyte repeatedly — the bound analytes can be desorbed by changing the pH of the solution. It is highly sensitive and it can detect molecules in the femtomolar range. Furthermore, the binding kinetics (affinity) between two molecules can be easily calculated (Bhunia *et al.*, 2007; Rasooly and Herold, 2006).

SPR-based sensors have been widely investigated for their suitability in the detection of foodborne pathogens (Leonard *et al.*, 2004). In most applications, this system shows promising results with toxins while signals with whole cells were inconsistent and, depending on the instrumentations, some are unable to detect whole cells. The literature on SE detection by SPR is abundant. Enterotoxin detection methods were optimized in different SPR systems. IAsys and BIACORE systems were used for the detection of SEA and SEB, respectively, in a sandwich immunoassay format at the 10–100 ng range when suspended in various complex food matrices including milk, hotdogs, potato salad, and mushrooms (Rasooly, 2001; Rasooly and Rasooly, 1999). In these methods, immobilized antibody on the chip surface was allowed to capture the toxins and then a second antibody was added to amplify the SPR signal. Naimushin *et al.* (2002) used a two-channel SPR system similar to the single channel Spreeta^{TM} for the detection of SEB. In the two-channel system, one channel served as a reference for real-time monitoring of the sample. Homola *et al.* (2002) reported a novel dual-channel SPR based on ''wavelength modulation'' for the detection of SEB in a direct binding-to-antibody or in a sandwich assay configuration. In the direct-binding assay, toxin bound to immobilized antibody on the chip surface was detected at 5 ng/ml, while in the sandwich assay format, a second antibody was used to amplify the signal, thus improving the sensitivity and the detection limit (0.5 ng/ml). A two-step approach of SPR and mass spectrometry was proposed for detection of SEB (Nedelkov and Nelson, 2003; Nedelkov *et al.*, 2000). In this method, an SPR system, Biacore X instrument, was used to capture SEB on the chip surface. Then the bound toxin was analyzed by the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and a concentration of 1 ng/ml of toxin suspended in milk or mushroom was detected. Besides enterotoxin, in a recent report, Subramanian *et al.* (2006a) used a Reichert SR7000 SPR to detect cells of *S. aureus* at a detection limit of 10^5 cfu/ml in a direct or a sandwich immunoassay configuration. A self-assembled monolayer

based on alkane monothiol and dithiol dendritic tether conjugated to anti-*S. aureus* antibody was used on the sensor chip surface for detection.

An SPR-based system was also used for the detection of *E. coli* O157:H7 cells with variable success. In the earlier published works, Medina and colleagues (Fratamico *et al.*, 1998; Medina *et al.*, 1997) employed the BIACORE system in a sandwich immunoassay format to detect *E. coli* O157:H7 cells with a detection limit of 5–7 $\times$ 10^7 cfu/ml. Oh *et al.* (2002) reported the detection of *E. coli* O157:H7 at a detection limit of 10^4 cfu/ml using an SPR spectroscope (Multiscop^{TM}, Optrel GbR, Germany) system. They used an 11-mercaptoundecanoic acid-mediated SAM of Protein G to immobilize *E. coli* O157:H7-specific MAb. Later, the sensitivity was improved by two orders of magnitude (10^2 cfu/ml) by employing a nano-fabrication strategy for monolayer assembly (Oh *et al.*, 2003). The same SPR set up was employed for the detection of multiple pathogens in a multiplex format using different specific antibodies to *E. coli* O157:H7, *Salmonella* Typhimurium, *Legionella pneumophilia*, and *Yersinia enterocolitica* (Oh *et al.*, 2005). However, the efficacy of the system to detect pathogens from food matrices or in presence of competing microflora was not verified. Su and Li (2005) used a miniaturized portable SPR, Spreeta^{TM}, to determine the sensitivity of the system to detect *E. coli* O157:H7 cells using a PAb as capture antibody and compared the data with a quartz crystal microbalance (QCM) immunosensor. The detection range for SPR was determined to be 10^5–10^8 cfu/ml, while it was 10^6–10^8 cfu/ml for QCM. Using Spreeta^{TM}, Meeusen *et al.* (2005) detected *E. coli* O157:H7 within 35 min and the limit of detection (LOD) was determined to be 8.7×10^6 cfu/ml. The sensitivity suffered when the target cells were mixed with heterogeneous bacterial cultures. The detection limit increased to 10^7 cfu/ml when the nontarget bacterial (*Salmonella* Typhimurium) concentration was 10^6 cfu/ml or less. The sensor response was further diminished when nontarget bacteria were present at 10^7 cfu/ml. In spite of such interference, they forecast that the technology could be used in the food industry for pathogen monitoring during HACCP implementation. Subramanian *et al.* (2006b) used mixed assembled monolayers consisting of polyethylene glycol-terminated alkanethiol to immobilize antibody for detection of *E. coli* O157:H7 in the Reichert SR7000 SPR biosensor. They used the direct and sandwich immunoassay formats to determine the LOD. The sandwich assay was found to be more sensitive (LOD = 10^3 cfu/ml) than the direct assay (10^4–10^6 cfu/ml). Using wavelength interrogation SPR, Taylor *et al.* (2005) were able to detect viable or heat-killed or detergent-lysed cells of *E. coli* O157:H7. They used a self-assembled monolayer of alkanethiol on a gold surface to immobilize MAb using EDC/NHS-coupling chemistry. Following the capture of cells, a second antibody was used to amplify the signals with a resulting

detection limit for lysed cells of 10^4 cells/ml, heat-killed cells of 10^5 cells/ml, and viable cells of 10^6 cfu/ml.

Koubova *et al.* (2001) reported the use of SPR for the detection of *Salmonella* Enteritidis at a detection limit of 10^6 cells/ml and concluded that the sensitivity was comparable with ELISA. The BIACORE 3000 system was used to monitor serum antibody to *Salmonella* Typhimurium and *Salmonella* Enteritidis during infection in chickens (Jongerius-Gortemaker *et al.*, 2002). Recombinant flagellar antigens were immobilized on the sensor chip, and the sera from experimentally infected or noninfected birds were tested for preharvest monitoring of pathogens. Bokken *et al.* (2003) used the same BIACORE system in a sandwich format to detect *Salmonella* group B, D, and E. They successfully detected 53 *Salmonella* serovars at 1×10^7 cfu/ml, and the background signal from various non-*Salmonella* organisms was well below the positive signals received from the target bacteria. Oh *et al.* (2004) employed the Multiscop^{TM} SPR to detect *Salmonella* Typhimurium at a range of 10^2–10^9 cfu/ml in a buffer system under an ideal laboratory set up. They used a SAM of 11-mercaptodecanoic acid with Protein G to immobilize antibody on the gold surface. Since they have not tested the sensor with food samples, it is difficult to speculate about the sensitivity of the sensor in the presence of inhibitors like food particles or natural microflora.

Literature on the application of SPR for *L. monocytogenes* detection is somewhat scanty. In an earlier proof-of-concept study, Koubova *et al.* (2001) were able to detect *L. monocytogenes* at 10^6 cells/ml without showing data on its relative selectivity or specificity. Later, Lathrop *et al.* (2003) employed a resonant mirror biosensor (IAsys) and demonstrated that this system can detect surface protein extracts from *L. monocytogenes* cells. A MAb to a 66-kDa protein was immobilized on the sensor surface by using EDC/NHS-coupling chemistry. The specificity of the assay depended on the cross-reactivity of the antibody. It showed reaction with *L. monocytogenes* and *L. innocua* and the signal from other *Listeria* spp. was equivalent to the background signal. The IAsys system failed to show any discernable signal from whole cells even at 10^8 cfu/ml, suggesting that this sensor configuration may not be suitable for the detection of whole cells (Lathrop *et al.*, 2003). Leonard *et al.* (2004) employed the BIACORE 3000 to detect *L. monocytogenes* cells at 1×10^5 cfu/ml through a subtractive inhibition assay using anti-*Listeria* PAbs and through stepwise removal of bound bacteria by allowing binding of anti-Fab antibody. More recently, using the same SPR system, and a protein-specific antibody (anti-Internalin B), the same group (Leonard *et al.*, 2005) was able to detect *L. monocytogenes* at 2×10^5 cells/ml. While the cross-reactivity of the anti-InlB antibody has been determined by ELISA inhibition assay, the specificity of the sensor with those cross-reactive bacteria has not been tested.

SPR systems also showed encouraging results with their ability to detect mycotoxins. The BIACORE was used to detect a mycotoxin, DON, produced by *Fusarium* species, from spiked wheat sample in a competitive inhibition assay (Schnerr *et al.*, 2002). Biotinylated DON was immobilized on the sensor chip which was previously coated with streptavidin. Mycotoxin extracts from wheat samples were first allowed to react with the antibody and then injected into the BIACORE. The detection range was established to be 0.13–10 μg/ml. In a slightly modified format, DON was also detected by SPR at a range of 2.5–30 ng/ml (Tudos *et al.*, 2003).

It is noteworthy that besides its potential use in pathogen detection, SPR is now widely used to study the molecular interaction of food-related bacteria with host cells. Uchida *et al.* (2004) used the BIACORE 1000 to analyze the adhesion characteristics of strains of *Lactobacillus acidophilus* to human A-type antigen or human colonic mucin for use as probiotics. Kim *et al.* (2006a) used the IAsys sensor to determine the binding affinity of a *Listeria* adhesion protein with its corresponding mammalian cell receptor, Hsp60, and demonstrated that the ligand–receptor interaction was highly specific.

C. Piezoelectric (PZ) biosensors

This sensor detects changes in the mass on the surface of a quartz crystal without the use of any labeling molecules. Antibodies are used for the specific binding of the analytes. Once bound, the complex increases the mass of the crystal, thereby changing the resonance frequency when an oscillating electric field is applied across the device. The frequency variation is measured by a quartz crystal analyzer (O'Sullivan *et al.*, 1999). SEs were detected at microgram quantities using this sensor (Harteveld *et al.*, 1997; Lin and Tsai, 2003). *Salmonella* Typhimurium cells were detected with a detection range of 9.9×10^5 to 1.8×10^8 cfu/ml (Park and Kim, 1998). Pathirana *et al.* (2000) reported an improved method with a detection limit of a few hundreds cells. They first immobilized the anti-*Salmonella* antibody on the quartz crystal by the Langmuir–Blodgett method and the resonant frequency was monitored by using a PM-700 Maxtek plating monitor with a frequency resolution of 0.5 Hz at 5 MHz. A variation of the PZ system is called a QCM, which consists of a thin quartz disc with implanted gold electrodes. QCM has been used for the detection of *L. monocytogenes* in the range of 2.5×10^5 to 2.5×10^7 cells/crystal (Minunni *et al.*, 1996), *Salmonella* Typhimurium at 1×10^5 to 5×10^8 cfu/ml (Wong *et al.*, 2002), *Salmonella* Paratyphi at 10^2–10^5 cfu/ml (Fung and Wong, 2001), *Bacillus cereus* at 10^4 cells/ml (Vaughan *et al.*, 2003), and *E. coli* O157:H7 at 10^3–10^8 cfu/ml (Su and Li, 2004). QCM has been shown to be effective for detection of *Mycobacterium tuberculosis*

from clinical specimens with a detection limit of 2×10^3 cells/ml (He *et al.*, 2003).

D. Electrochemical immunosensor

Electrochemical immunosensors are an extension of conventional antibody-based enzyme immunoassays (ELISA) where catalysis of substrates by an enzyme conjugated to an antibody produces products in the form of pH change, ions, and oxygen consumption that generate electrical signals on a transducer (Warsinke *et al.*, 2000). Potentiometric, capacitive, and amperometric transducers have been used for such applications. In amperometric detection, for example, alkaline phosphatase (AP) conjugated to an antibody hydrolyzes *p*-nitrophenyl phosphate to phenol, which is detected by voltammetry. In light-addressable potentiometric sensors (LAPS), urease-conjugated antibody hydrolyzes urea, resulting in the production of carbon dioxide and ammonia that changes the pH of the solution (Fig. 1.3). A silicon chip coated with a pH-sensitive insulator and an electrochemical circuit measures the alternating photocurrent as a light emitting photodiode shines on the silicon chip. These sensors are very

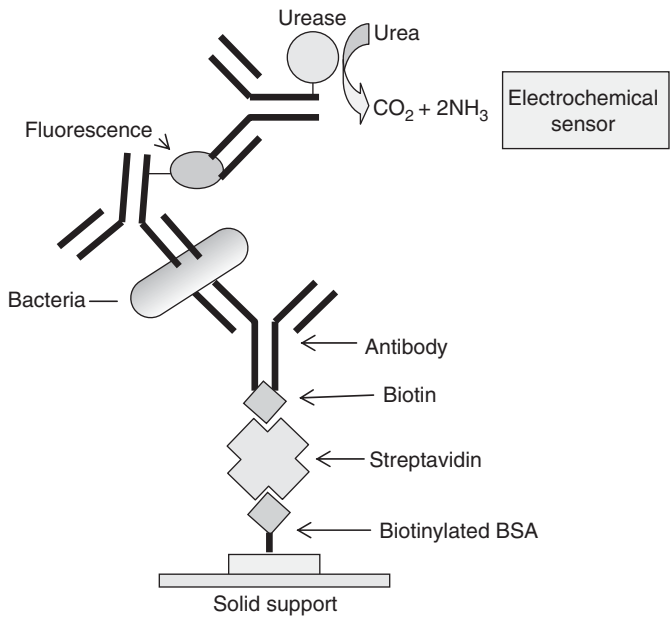

FIGURE 1.3 Schematic drawing of a light addressable potentiometric sensor (LAPS) for pathogen detection. (Adapted from reference Gehring *et al.*, 1998).

sensitive and have been used for detection of *Salmonella* and *E. coli* O157:H7 in 30–90 min.

Light-addressable potentiometric sensors (LAPS) have been used for the detection of *E. coli* O157:H7 (Gehring *et al.*, 1998; Tu *et al.*, 1999). In this assay configuration (Fig 1.3), capture antibody was first immobilized on a membrane or magnetic beads for the capture of target cells. In a sandwich format, a fluorescent-labeled antibody was allowed to bind to the target cells. Urease-labeled anti-fluorescent antibody was then added. In presence of urea, NH_3 was produced that changed the pH of the solution on the n-type sensor coated with a pH sensitive insulator that recorded the voltage change. The detection limit was reported for such sensor to be 7.1×10^2 cells/ml for heat-killed cells and 2.5×10^4 cells/ml for live cells. Experiments with food samples showed that *E. coli* O157:H7 at 1 cfu/g of hamburger could be detected after 6 h of enrichment (Tu *et al.*, 1999, 2002). LAPS was also demonstrated to be suitable for detection of BWA, *Y. pestis* and spores of *Bacillus subtilis* (globigii) (Dill *et al.*, 1997; Uithoven *et al.*, 2000). A potentiometric alternating biosensing (PAB) system based on LAPS was also used to detect *E. coli* cells at 10 cells/ml in vegetables such as lettuce, carrots, and rucola in about 1.5 h (Ercole *et al.*, 2002, 2003). This method was employed for the quality verification of fresh produce. A disposable electrochemical sensor using conductive polyaniline in a sandwich configuration was used to detect *E. coli* O157:H7 in fresh produce including alfalfa, lettuce, sprouts, and strawberries (Muhammad-Tahir and Alocilja, 2004). A silicon chip-based LAPS was employed for detection of *Salmonella* Typhimurium at concentrations of 119 cfu (Dill *et al.*, 1999). In this case, the pH change during catalysis of urea with urease was sensitively detected using a silicon chip.

An immunoelectrochemical sensor has been used for the detection of *E. coli* O157:H7 cells from buffer (Brewster and Mazenko, 1998; Ruan *et al.*, 2002a). Target bacteria were mixed in a solution with AP-labeled antibody and captured on a membrane. The addition of substrate, *p*-aminophenyl phosphate (*p*-APP), produced an electroactive product, *p*-aminophenol (*p*-AP), which was detected by the BAS 100B/W Electrochemical Analyzer (Brewster and Mazenko, 1998). The sensitivity was estimated to be 5×10^3 cells/ml and the assay time was 25 min. Later, the assay was modified where bacteria were first captured by immunomagnetic beads, and then reacted with AP-conjugated antibody followed by reaction with the substrate, 1-napthyl phosphate (1-NP), a substitute for *p*-APP, resulted in an improved signal (Gehring *et al.*, 1999). This system was able to detect *E. coli* O157:H7 at 4.7×10^3 cells/ml in a porcine carcass wash within 80 min. Essentially using the similar antibody capture strategy on magnetic beads and AP-labeled detection antibody, Gehring *et al.* (2004) described an enzyme-linked immunomagnetic chemiluminescence assay (ELIMCL) for detection of *E. coli* O157:H7 in 75 min with a detection

limit of 7.6×10^3 cfu. Addition of the chemiluminescence substrate, APS-5, for AP resulted in luminescence, which was measured in a FB-12 Luminometer (Zylux, Oak Ridge, TN). This system allowed detection of the pathogen in ground beef with initial cell concentrations of 10 cfu/g after only 5.5 h of enrichment. Electrochemical immunoassay with immunomagnetic bead-captured bacteria as described above was also used for detection of *C. jejuni* from chicken carcasses with a detection limit of 2.1×10^4 cfu/ml (Che *et al.*, 2001). A menadione-catalyzed luminol chemiluminescence assay was developed for rapid quality assessment of food products for generic *E. coli* (Kawasaki *et al.*, 2004). The assay principle is based on the catalysis of active oxygen (O_2^- and H_2O_2) produced by aerobic respiration of viable cells with menadione. The addition of luminol produced chemiluminescence that was detected by a luminometer. The system was able to detect *E. coli* at 1–10 cfu/ml in milk, vegetable juice, green tea, and coffee after 7 h of enrichment.

An electrochemical chemiluminescence biosensor for *Salmonella* Typhimurium was described (Varshney *et al.*, 2003). In this method, they collected bacteria captured on immunomagnetic beads and then reacted with horseradish peroxidase (HRP)-labeled antibody. The addition of luminol produced a chemiluminescence signal (mV) that was collected through a fiber optic light guide. The detection limit was established as 1.97×10^3/ml. The sensor was highly specific for *Salmonella* Typhimurium and showed significantly higher signals than for other serovars of *Salmonella* and other common foodborne bacteria such as *Citrobacter freundii*, *Pseudomonas aeruginosa*, *L. monocytogenes*, *C. jejuni*, and *E. coli*. A highly specific chemiluminescence sensor was also reported for *E. coli* O157:H7 using the same basic configuration as above except the antibody was highly specific (Ye *et al.*, 2002). The reported detection limit was 1.8×10^2 cfu/ml with a signal output value of 3.8 mV. These assays could be completed in 90 min.

A disposable electrochemical enzyme-amplified genosensor was described for specific detection of *Salmonella* (Del Giallo *et al.*, 2005). A DNA probe specific for *Salmonella* was immobilized onto screen-printed carbon electrodes and allowed to hybridize with a biotinylated PCR-amplified product of *Salmonella*. The hybridization reaction was detected using streptavidin conjugated-AP where the enzyme catalyzed the conversion of electroinactive α-naphthyl phosphate to electroactive α-naphthol, which was detected by differential pulse voltammetry.

The monitoring of oxygen consumption during active bacterial growth in medium was measured by electrochemical cyclic voltammetry as a means to detect the growth of live cells (Ruan *et al.*, 2002b). In this method, *Salmonella* Typhimurium growth in a selective medium was shown to be inversely proportional to the rate of oxygen consumption. A linear response was observed for cell concentrations of $1–2 \times 10^0$ to

1–2 $\times$ 10^6 cells, and the growth was detected at 10 h and 2.1 h, respectively. Though it was claimed to be more sensitive than PCR or immunoassay, it is highly unlikely to be specific because the specificity directly depends on the selectivity of the medium used.

An electrochemical sensor using an array microelectrode was tested for the detection of allergens such as mite and cedar pollen (Okochi *et al.*, 1999). Blood was used in the assay and the release of serotonin, a chemical mediator of allergic response, which is electrochemically oxidized at the potential around 300 mV, was monitored for electrochemical detection by cyclic voltammetry.

E. Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) allows the measurement of FRET during interaction of two molecules labeled with two different fluorophores whose absorbance and emission spectra overlap. One fluorophore acts as a donor (reporter) and the other one as an acceptor (quencher) (Majoul, 2004). The FRET-based assay is considered as a homogeneous ''one-step'' assay without the requirement for a washing step. The application of FRET in pathogen detection has been successfully demonstrated. Bruno *et al.* (2001) used a cuvette-based spectrofluorometer to detect *B. cereus* spores (1–2.5 $\times$ 10^5/ml) and *E. coli* cells (3.5 $\times$ 10^5/ml) in 30 min. They used Oregon Green 514 (OG-514)-labeled antibody and QSY-7-labeled spores of *B. cereus* or vegetative cells of *E. coli* O157:H7. When OG 514-labeled antibody binds to QSY-7-labeled cells or spores, OG-514 activity is quenched due to proximal location of QSY-7. The introduction of unlabeled spores or bacteria causes antibody detachment due to the equilibrium shift and the antibody binds to unlabeled cells which results in an increased fluorescence activity due to separation of the reporter from the quencher (Bruno *et al.*, 2001). Employing another pair of fluorophores (Alexa Fluor 546 and Alexa Fluor 594), Ko and Grant (2003) demonstrated that *Listeria* or *Salmonella* antigens could be successfully detected using FRET. Very recently, they (Ko and Grant, 2006) used FRET-based fiber optic sensor for the detection of *Salmonella* Typhimurium cells at 10^3 cfu/g in a homogenized pork sample. A FRET-based PCR assay was also employed to detect *L. monocytogenes* from a model food — nonfat dry milk at concentrations of 10^3–10^4 cfu/25 ml of sample (Koo and Jaykus, 2003). In this assay, a single internal oligonucleotide probe labeled with fluorescein reporter dye at the 5′-end and a 3′ DABCYL-labeled quencher dye was used to amplify the *hly* or *iap* gene. During amplification of the target DNA, the reporter is freed from the quencher due to the endonuclease activity of the *Taq* DNA polymerase and the release of the reporter (fluorescence activity) was proportional to the rate of amplification of the target.

F. Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) spectroscopy is used to generate bacterial spectral scans based on the molecular composition of a sample. Basically, infrared spectroscopy consists of the infrared source, the sample, and the detector. When IR is absorbed or transmitted through the sample to the detector, it generates a ''scan'' or ''fingerprint'' profile. A library of spectral scans can be generated for different bacterial species and strains, which can be used for future comparison. This method requires transfer of cells (biomass) from the growth media to an IR-reflecting substrate for spectral collection. It is a nondestructive rapid method and sample identification depends on the available spectral library. FT-IR has been used for classification or identification of several foodborne pathogens: *Yersinia*, *Staphylococcus*, *Salmonella*, *Listeria*, *Klebsiella*, *Escherichia*, *Enterobacter*, *Citrobacter*, etc. (Gupta *et al.*, 2005; Mossoba *et al.*, 2005; Sivakesava *et al.*, 2004). FT-IR photoacoustic spectroscopy was used for the identification of spores of several *Bacillus* species with 100% accuracy (Thompson *et al.*, 2003). The same system was also used for the identification of *E. coli* O157:H7 on an apple surface (Irudayaraj *et al.*, 2002). The FT-IR system equipped with a focal plane array (FPA) detector allowed the rapid and precise identification of several bacterial species including *S. aureus*, *Y. enterocolitica*, *Klebsiella pneumoniae*, *E. coli*, *C. freundii*, *Salmonella* Typhimurium, and *Enterobacter cloacae* (Kirkwood *et al.*, 2004). Recently, a comprehensive analysis of *S. aureus* strains isolated from milk or milk products was done using FT-IR (Lamprell *et al.*, 2006). Furthermore, this method allowed successful discrimination of *S. aureus* from other staphylococcal species.

Lipopolysaccharide extracts from different pathogenic and nonpathogenic *E. coli* strains were also analyzed by FT-IR with principle component analysis and canonical variate analysis (Kim *et al.*, 2006b). The data showed that *E. coli* strains can be discriminated with >95% accuracy. *Listeria* species were also reliably classified by FT-IR coupled with an artificial neural network technology with a success rate of 96% (Rebuffo *et al.*, 2006), while the identification rate for *L. monocytogenes* alone was 99.2%.

G. Light scattering

Light scattering dates back many decades and has been used for many years in the semiconductor industry for the monitoring of defects on wafers. Light scattering technology differentiates samples based on refractive index, size, shape, and composition. When an illuminated light from a polarized monochromatic light source shines on a sample (bacteria, for example), scattered light forms distinct patterns which

could be used for identification and detection of bacteria. However, stage of growth, growth medium, growth temperature, aeration, and the final dilution of the suspended medium can affect the reproducibility of this method. Fraatz *et al.* (1988) employed multiparameter light scattering to detect culture contamination, which is of great importance to the fermentation industry. They were able to detect the contamination of *E. coli* with *B. thuringiensis* at levels of 1% or higher. Bronk *et al.* (1995) used polarized light scattering to measure the diameter of rod-shaped bacteria and later, Van De Merwe *et al.* (1997) determined the effect of nutrients on the diameter of rod-shaped bacteria. The same group also examined the effect of metal toxicity on *E. coli* cells using light scattering (Bronk *et al.*, 2001). Differential polarization light scattering (DPLS) was used to differentiate spores of *B. subtilis* (Diaspro *et al.*, 1995). It is highly sensitive and it can discriminate between two different strains of *B. subtilis*. DPLS has been used for the characterization of microorganisms in suspension (Bronk *et al.*, 2001; Perkins and Squirrell, 2000; Wyatt, 1969). However, there are challenges associated with bacteria in suspension, such as the purity of cultures and the arrangement of cells which appear in chains or clusters. The orientations of and distances between cells change with time. Therefore, an averaging method to account for the relative orientation and movement is needed. However, a colony on a solid surface such as agar is more stable and its optical response could be modeled with scalar diffraction theory. The optical back-scattering method is widely used for wafer inspection and for studying biological cells (Hielscher *et al.*, 1997; Jordan *et al.*, 2002), but it did not produce reproducible results when tested with bacterial colonies (Nebeker *et al.*, 2001). Conversely, optical forward scattering yielded reproducible scattering patterns. Recently, a diode laser was used to generate light scattering images of *Listeria* colonies growing on agar plates for their identification and classification (Banada *et al.*, 2007; Bayraktar *et al.*, 2006. Fig. 1.4). The scatter images of bacterial colonies were characterized using Zernike moment invariants, and principal component analysis and hierarchical clustering were performed on the results of feature extraction. The system was able to distinguish different species of *Listeria* with 90–100% accuracy and could be used in a simple and noninvasive manner to characterize bacterial colonies on agar plates (Banada *et al.*, 2007).

H. Impedance-based biochip sensor

The concept of impedance microbiology is more than a century old; however, it gained its popularity only in the mid-seventies. Impedance is based on the changes in conductance in a medium due to the microbial breakdown of inert substrates into electrically charged ionic compounds and acidic by-products. The detection time, that is, the time necessary for

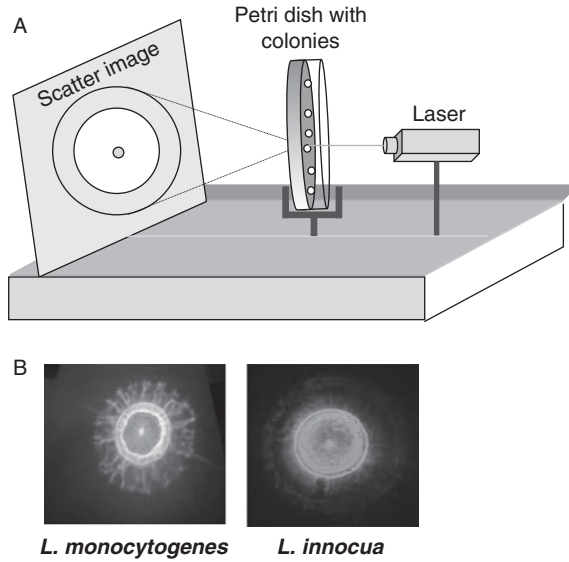

FIGURE 1.4 Optical light scattering for detection of bacterial colonies grown on solid agar plates. (A) Forward light scattering device and (B) scatter images of colonies of *L. monocytogenes* and *L. innocua*. (Adapted from references, Bayraktar *et al.*, 2006; Banada *et al.*, 2007.)

these changes to reach a threshold value, is inversely proportional to the initial inoculums and the physiological state of the cells. The principle of all impedance-based systems is that they measure the relative or absolute changes in conductance, impedance, or capacitance at regular intervals (Fig. 1.5). In media-based impedance methods, bacterial metabolism results in increased conductance and capacitance, with decreased impedance (Ivnitski *et al.*, 1999). The major advantage of this system is that it allows the detection of only the viable cells, which is the major concern in food safety. The basic technical equipment required for performing impedance microbiology consists of special incubators and their culture vessels (equipped with electrodes) and an evaluation unit with computer, printer, and appropriate software.

Microbial metabolism results in an increase in both conductance and capacitance causing a decrease in impedance and a consequent increase in admittance. In the Rapid Automated Bacterial Impedance Technique (RABIT) system, the admittance was plotted against time to provide results (Bolton, 1990). The final electrical signal is frequency- and temperature dependent and it has a conductive and a capacitive component. At present, impedance instruments are able to detect 10^5–10^7 bacteria/ml (Ivnitski *et al.*, 2000). Several commercially available systems are operated

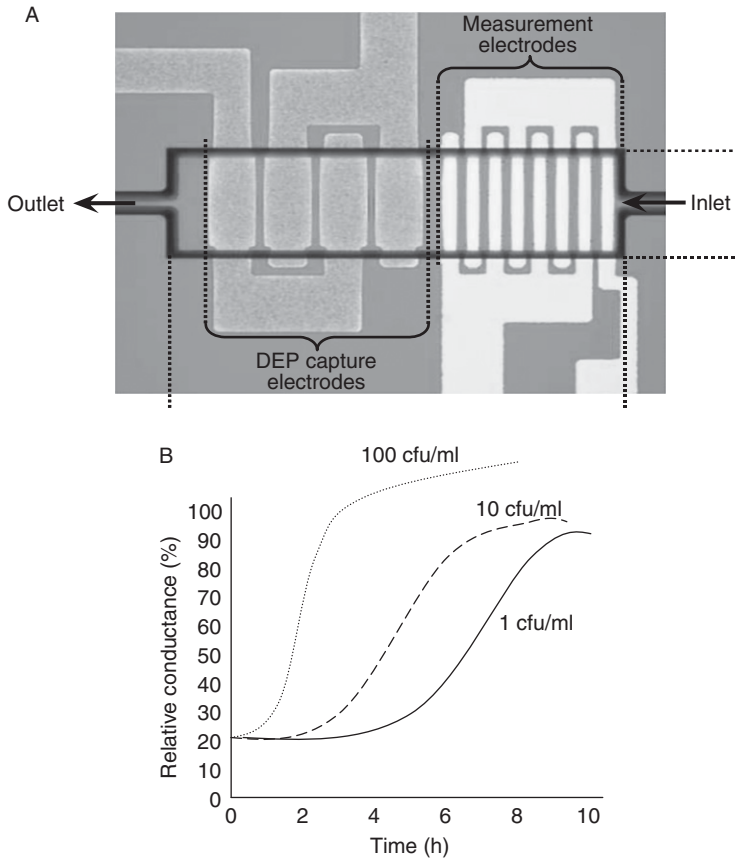

FIGURE 1.5 Impedance-based detection of bacterial growth on microfluidic biochip. Bacterial cells are first captured by applying DEP force on chip and then the bacterial cells are allowed to grow. Bacterial growth and utilization of inert substrates into charged ionic species will then change the conductivity of the solution. Panel (A) is showing a prototype chip fabricated by Bashir and his colleagues (Chang *et al.*, 2002; Li and Bashir, 2002) with interdigitated electrodes for DEP capture of bacteria and the interdigitated measurement electrodes to monitor growth. Panel (B) is showing a conductance plot generated based on bacterial growth of arbitrary numbers of colony forming units (cfu)/ml.

based on the impedance measurement. The Bactometer® (bioMérieux, Marcy l'Etoile, France) has the capacity of testing up to 512 samples resulting in total microbial counts in 6–24 h and more specific counts (yeast, mold, lactic acid bacteria, etc.) in 24–48 h. The Malthus AT analyzer (Malthus Instruments, Bury, UK), BacTrac™, and μ-Trac microorganism growth analyzer (SyLab, Purkersdorf-Vienna, Austria) are used

for bacterial growth measurement. Quicker results can be obtained in the impedance method by using it in conjunction with an IMS step to initially concentrate the bacteria (Safarik *et al.*, 1995).

The bacterial contamination in milk has been analyzed using the Bactometer for quality assessment purposes (Madden and Gilmour, 1995). For specific detection of *Listeria*, this system was used with a high conductive *Listeria* electrical detection (LED: conductivity >2 mS) medium and *Listeria*-selective supplements. It took about 30 h to induce a 30% change in capacitance (Capell *et al.*, 1995). The LED medium was also successfully applied for the detection of *Listeria* in cheese samples (Rodrigues *et al.*, 1995). The impedance-based assay has also been used for the detection and enumeration of *Campylobacter* (Falahee *et al.*, 2003), *E. coli* (Colquhoun *et al.*, 1995; Upadhyay *et al.*, 2001), *Staphylococcus* (Glassmoyer and Russell, 2001), and *Salmonella* (Fehlhaber and Kruger, 1998; Yang *et al.*, 2003) from food samples. The impedance method has been accepted by the Association of Official Analytical Chemists, Intl., (AOAC) as a first action method (Gibson *et al.*, 1992).

To improve sensitivity, and to overcome limitations posed by above technologies, microelectronics or microfabricated electronic devices such as a semiconductor chips referred to as ''biochips'' are used. In this biology-based microelectrical-mechanical systems (Bio-MEMS) device, the cells are confined into a very small volume with rapid turnover of substrate into electrically charged by-products resulting in rapid and sensitive detection of bacterial growth by impedance measurement. The biochip is a microfabricated silicon device (microchip) with interdigitated electrodes, which can detect a few bacterial cells in nanoliter volumes (Gomez *et al.*, 2001, 2002). One hundred bacterial cells confined into a volume of 10 nl results in a concentration of 10^7 cells/ml are detected in the device. *L. monocytogenes* cells were successfully detected at <10 cells/5.27 nl chamber with only a 2-h incubation step (Gomez *et al.*, 2002). Even the presence of heat-killed bacterial cells did not interfere with the signal. Further on-chip separation of live bacterial cells from dead cells by employing a DEP force allowed strategic localization of cells in different compartments on the biochip (Chang *et al.*, 2002; Li and Bashir, 2002; Fig. 1.5). Immobilized antibody on the chip surface allowed specific capture of DEP-driven *L. monocytogenes* cells for impedance-based detection (Yang *et al.*, 2006). The sensitivity of the impedance-based measurement was improved manyfold with a detection limit of 10–1000 cells/ml by introducing a newly formulated low conductive growth medium (LCGM) into the chip (Banada *et al.*, 2006; Yang *et al.*, 2005). In addition, bacterial capture on the biochip was facilitated by employing various specific and nonspecific means such as lysozyme (Huang *et al.*, 2003a,b), functionalized polystyrene bead-based, or avidin–biotin-based antibody immobilization methods (Huang *et al.*, 2003c).

Wang *et al.* (2006) described a flow-through microfluidic biochip that allows on-chip lysis of cells for detection of intracellular proteins or nucleic acids using an antibody or DNA probe labeled with fluorescence dye for pathogen detection. Much of the success with biochip detection also depends on the sample preparation to prevent food particle contamination. Employing appropriate membrane separation technologies with micron-sized pores, the clarified samples can be delivered to the biochip for growth monitoring by impedance measurement (Chen *et al.*, 2005a,b).

Ruan *et al.* (2002a) reported an impedance immunosensor method for detection of *E. coli* by immobilizing an anti-*E. coli* antibody on the electrode surface and applying an impedance measurement. The recognition process was further amplified by the coupling of the *E. coli* antibody-AP conjugate to the surface that stimulated the precipitation of an insoluble material onto the electrode surface. This impedance immunosensor allowed analysis of the *E. coli* O157:H7 cells with the detection limit of 6×10^3 cells/ml. Suehiro *et al.* (2003) used the agglutination of target bacteria through an antibody-antigen reaction to separate *E. coli* from *Serratia marcescens* by using dielectrophoretic impedance measurement.

I. Cell-based sensor

The detection systems that incorporate whole cells or cellular components have a distinct advantage of responding in a manner that can offer insight into the physiological effects of an analyte in minutes (Pancrazio *et al.*, 1999). Mammalian cell-based biosensors take advantage of natural cellular fluorescence, metabolism, impedance, and intracellular and extracellular potentials to gather data in reference to a test sample. An advantage to biosensors employing mammalian cells is that they provide additional information as to the possible physiological effects of the sample according to their biochemical or physiological response. Cell-based assays (CBAs) continue to serve as a reliable method to probe for the presence of pathogens in clinical, environmental, or food samples (Stenger *et al.*, 2001; Ziegler, 2000. Fig. 1.6). The CBA systems can report perturbations in the ''normal'' physiological activities of mammalian cells as a result of exposure to an ''external'' or environmental challenge (Ziegler, 2000). Some of the CBAs utilize the metabolic responses of cells (like cyanobacteria) to detect biological products, like oxygen and herbicides in water (Rawson *et al.*, 1989).

In another type, mammalian cells or plasma membranes are used as electrical capacitors. Electrical impedance (EI) uses the inherent electrical properties of cells to measure the parameters related to the tissue environment (Kyle *et al.*, 1999). The mechanical contact between cell–cell and cell–substrates is measured via conductivity or EI (Deng *et al.*, 2003;

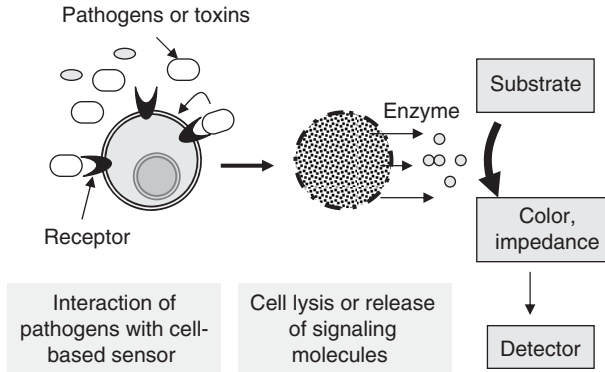

FIGURE 1.6 Schematic representation of cell-based sensor (CBB) for pathogen detection. After binding to receptor on mammalian cells, pathogen or toxin will aid in the release of signaling molecules such as fluorescence or enzyme that can be detected using an appropriate sensor.

Giaever and Keese, 1991, 1993). The cell can be equated to a simple circuit since it is nothing more than conductive fluid encapsulated by a membrane surrounded by another conductive fluid. The conductive fluids make up the resistance elements of the circuit, while the membrane acts as a capacitor. Impedance methods have been used to monitor tissue cultures online and in real time. Cells derived from a male monkey's kidney cells were adherently grown on interdigitated electrode structures (IDES) (Ehret *et al.*, 1996). Changes in impedance were able to detect changes in cell density, growth, or cellular behavior. These biosensors are able to provide detailed information about the growth characteristics of the tissue culture, including information on spreading, attachment, and cellular morphology. Furthermore, any external factors such as live bacteria or active cytotoxins that affect the integrity of the membrane will alter the conductivity and, thus, will provide a signal. An interdigitated microsensor electrode was employed to detect the cytotoxic action of *L. monocytogenes* on macrophage cell line. Although positive signals were obtained, the reproducibility of the assay was unsatisfactory (Gray, 2004).

Also, mammalian cells are used to measure biochemical and metabolic end-products (delivered from cultured cells to the medium) (Ziegler, 2000). The CBAs can also measure the direct electrical response of electrogenic cells (neural cells, heart muscle cells, pancreas beta cells) or a neural cell network (Ziegler *et al.*, 2000). For example, neurons were used with great sensitivity to sense cell death dynamics, receptor–ligand interactions, alterations in metabolism, and generic membrane perforation processes. Neurons were shown to be an excellent sensor system to monitor their chemical environment and generate typical responses that are concentration- and substance specific (Gross *et al.*, 1992).

Cytosensor® Microphysiometer technology has been used to detect perturbation in mammalian cells (Hafner, 2000). The system measures small changes in extracellular acidification using a light addressable potentiometric sensor. If the metabolism is interfered with, acid excretion will be affected which could be sensitively measured by LAPS. In principle, this system should be suitable for monitoring pathogen interaction with mammalian cells.

Mammalian cells have been widely used for the analysis of the pathogenic potential of foodborne bacteria (Bhunia and Wampler, 2005). For *L. monocytogenes*, various cell types were used such as the enterocyte-like Caco-2 (Pine *et al.*, 1991; Van Langendonck *et al.*, 1998), macrophage (Dallas *et al.*, 1996), epithelial HT-29 (Roche *et al.*, 2001), and hybrid lymphocyte-Ped-2E9 (Bhunia *et al.*, 1994, 1995). Early work reported that a multiplicity of infection (MOI) of 100:1 (*L. monocytogenes*:hybridoma) resulted in 85% cell death in 4 h, as measured by the Trypan blue exclusion assay. Later, using the colorimetric lactate dehydrogenase (LDH) enzyme or AP release assays, the same system produced 80–95% cytotoxicity in 4–6 h, in which enzyme release correlated directly with the Trypan blue exclusion assay (Bhunia and Westbrook, 1998). *L. monocytogenes* induced severe membrane pore formations and cell death was shown to be due to the induction of apoptosis (Bhunia and Feng, 1999; Menon *et al.*, 2003). Sulfhydryl-activated listeriolysin O (LLO) is the primary factor responsible for cell lyses, thus addition of a thiol-reducing compound, dithiothreitol (DTT), to the assay mixture expedited the assay where the cytotoxic effect was determined in 1.5–2 h (Westbrook and Bhunia, 2000). This assay is specific and sensitive in distinguishing virulent from avirulent species of *Listeria.* The assay time was again shortened to 1 h when a fluorescence-based cytotoxicity assay was incorporated (Shroyer and Bhunia, 2003). This hybridoma cell-based assay was used for detection of *L. monocytogenes* from food samples using a two-step method of immunobead capture and cytotoxicity analysis (Gray and Bhunia, 2005). Recently, work from our laboratory has shown that enterotoxigenic *Bacillus* species can be detected using the same Ped-2E9 cell-based cytotoxicity assay (Banerjee *et al.*, 2007; Gray *et al.*, 2005). The Ped-2E9 cell cytotoxicity assay is considerably faster than the other conventional CBAs like the MTT (3-[4,5-dimethyl thiazolyl-2]-2, 5-diphenyltetrazolium bromide)-based CHO cell assay, where cytotoxic effect was determined in just 1 h. The sensitivity of Ped-2E9 hybridoma cells to pathogenic *Listeria* and *Bacillus* species makes this cell line a potential candidate to be employed in a cell-based biosensor device.

A novel genetically engineered B-cell-based sensor was developed to detect various pathogens relevant to food safety and biowarfare agents (Rider *et al.*, 2003). The B-cells were engineered to express cytosolic aequorin, a calcium-sensitive bioluminescent protein, and pathogen-specific

surface antibodies. The assay was able to detect 500 cfu of *E. coli* O157:H7, 1000 spores of *B. anthracis*, and 200 cfu of *Y. pestis* in minutes. Banerjee *et al.* (2005) proposed a simple filtration tube-based cell sensor with a portable colorimetric detector (Ocean Optics spectrophotometer), where the Ped-2E9 cell line was immobilized in a gelatin matrix. Upon addition of a toxin preparation from *Listeria* or *Bacillus*, the Ped-2E9 cells released ALP and converted colorless *p*-nitrophenyl phosphate into a chomogenic product, which was detected by the spectrophotometer. Zhao *et al.* (2006) used a lipid bilayer (liposome) with an embedded cholesterol receptor for determining the action of LLO in an artificial cell-based biosensor. The liposome was immobilized in silica nano-composite materials (sol-gel) and the action of the toxin was quantified by measuring the release of a fluorescence reporter from liposome. The advantages of this system are that the liposome could be stored in ambient conditions for up to 5 months, and this system can detect nanogram quantities of toxin rapidly (in 30 min).

A mammalian cell-based biochip for allergen detection was also proposed (Matsubara *et al.*, 2004). They cultured a basophilic mast cell line (RBL-2H3) that releases histamine upon activation by an allergen, in a microfluidic biochip molded with PDMS (poly-dimethylsiloxane). The mast cells were pretreated with a fluorescent dye, quinacrine, that binds to histamine in acidic compartments of the cell granule by mass action. Upon exposure to the allergen, the IgE-activated cells release histamine along with the fluorescent dye, which is measured by a photomultiplier tube attached to a microscope.

IV. CONCLUSIONS

Many of the sensor platforms discussed above demonstrated the proof-of-concept by testing with pure bacterial cultures or toxins; however, only a handful of those were tested thoroughly with real-world food samples for their ruggedness, robustness, sensitivity, and cross-reactivity. Future efforts should focus on continued improvement in the applicability of some of those most promising sensors for their ability to detect foodborne pathogens from real-world food and environmental samples. Furthermore, the cost of the sensors should be addressed. Although at this time, it may not be a cost-effective technology, with continued advancement and mass production, the sensors should one day be affordable for the routine testing of samples. Sensors are effective only when a clean sample is delivered to the device. In spite of tremendous progress in sensor development, efforts to improve sample processing and preparation are lagging behind, which should be given a high priority to fully appreciate the usefulness of sensor technologies. Sensor technologies are

also becoming a powerful tool in biomedical applications in probing the human body for early diagnosis of malignant cancer, cardiovascular disease, diabetes, etc. If we continue to embrace this exciting field of science and technology, one day this will be integral part of human life and the way we live.

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